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Radegen Biotechnology

Creative Work: Clarification and correction of misstated concepts in the Theta+ N Liquid Transfer System as published on 03/08/2022

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Scientific concepts are difficult to comprehend and to explain due to their complex nature with little to no point of reference to anything learned in a classical western education since the science of molecular biology is very new. There are several other factors that contribute to the writing of vague or misstated descriptions of processes. At times, a paragraph that seems to be universally understood by participants of a development process is later revised due to room for making a point more clearly written. Here an attempt to describe the liquid handling of synthesis coupling reactions using an automated liquid handling robot is attempted.

As published at: https://archive.org/details/2022.-theta-n-liquid-transfer-system-for-polymer-including-dna-and-rna-synthesis/page/n1/mode/2up

DNA is composed of single stranded polymers of nucleotide monomers. Nucleotide are bound together in strings by phosphodiester bonds between the 3rd carbon of the deoxyribose ring and the 5th carbon of the next nucleotide deoxyribose ring. There are 4 types of nucleotide monomers that can canonically exist in a DNA molecule, adenine (a), thymine (t), cytosine (c) and guanine (g). The sequential composition of nucleotide in a DNA molecule is the primary basis of information storage for processing by a cellular infrastructure to perform some type of work such as enzyme production or cellular regulation. Recent advances in computer aided system design, also referred to as *in silico* design in biology have facilitated the production of synthetic DNA constructs in sizes as small as 25 bp to several thousand bp by enzymatically stitching together chemically synthesized oligonucleotides. Advances in chemical oligonucleotide synthesis processes have allowed the production of oligos up to 350 nt in length.

Radegen Biotechnology's RadegenBio+ DNA Forging system is a novel system designed to develop ssDNA oligonucleotides enzymatically instead of chemically using a template independent terminal polymerase. This technology was historically used to label the 3' end of an oligonucleotide with a biotin modification, a process termed "Pierce™ Biotin 3' End DNA Labeling". In the Pierce system a nucleotide with a 3' biotin modification is added to the 3' end of a ssDNA fragment with a deoxynucleotidyl terminal deoxy transferase (Dtd) enzyme. Additional nucleotides are not aberrantly added since the biotin modification blocks the 3' carbon. It seemed obvious to me that the use of nucleotides with a reversable 3' polymerization terminator could be used to generate DNA *de novo* molecule with a user defined sequence in a template independent manner similar to how an oligo is chemically synthesized.

There are two major considerations to be made when designing this novel DNA synthesis method, the enzymatic handling of the reaction (a topic that has been explained in other Creative Commons publications) and the liquid handling component of the reaction. There are several options that can be explored and those include using microfluidics for a lithographic process, designing a new dedicated instrument (a task that is out of scope for the company) and using a commercially available option for liquid handling work. The use of a robotic liquid handling device was chosen as the preferred method for process development since it is a solution that is widely available. Radegen Biotechnology is currently in talks with a major liquid handling robotics manufacturer to develop computer script to handle the process. The use of a robotic liquid handling technology would not be possible without the Theta+ N Liquid Transfer System for Polymer Synthesis Reactions Conducted in a 96 Well Plate creative concept. In this publication a process where 3-4 wells are addressed every time the robotic arm goes from a reagent reservoir to the reaction plate is described.

One of the misstated sentences describes DNA "consisting of 4 nucleotides". What is meant here is that in DNA every position within a polymer can consist of one of 4 nucleotides. Right after this misstatement I describe how this property allows for an exact prediction of the total number of molecules that can be determined by considering the total number of possible monomers in a position as a factor of polymer length. For example, for a DNA polymer with 3 monomer units there are 64 total number of molecules possible each with a unique combination of monomer sequences. A DNA polymer with 4 monomers units has 256 possible unique combinations and a polymer with 5 monomer units has 625 possible combinations. This number is calculated with the following formula: N^{p} (N^P), where N = the number of positions in a monomer and P = the total number of unique monomers possible at any given position. The property of a polymer with a small number of defined position variants (in the case of DNA a, t, c, or g) can be used to transfer liquid from a master microtiter plate with polymerization reagents to a DNA polymerization reaction plate 3 or 4 wells at a time while maintaining the sequence fidelity of the unique user defined molecule being assembled in each well. In cases where 3 wells are to be added at a time, liquid transfer events occur from a master plate containing individual wells with either a, t, c or g. The identity of a monomer in a well will depend on the wells position since the goal is to generate a pattern where every 3 bp combination is represented in three sequential wells in a row or column instead of having 3 unique monomer in one well. See image below for a visual example. Please see 2022.03.20-theta.ori-robo-3x.-cc-by-nc-sa-4.0 file as found on the IP property page of the website.

A multichannel pipet arm that is modified for only transferring liquid from 3 pipet tips at a time or by a handheld multichannel pipettor modified for the same task can be used to transfer liquid from the master plates to the reaction plate while maintaining the required sequence fidelity in each well. The example below has individual monomers serialized from the left to right of the plate and thus addition to the reaction plate would occur from three sequential wells in a row from a master plate to 3 sequential wells in a row in a reaction plate. So for example lets say that in well 1a you are synthesizing the gene that codes for ampicillin, in well 2a you are synthesizing the gene for alkaline phosphatase, and in well a3 you are synthesizing the gene that codes for Green Florescent Protein. The first nucleotide for each gene can either be different or the same, for this example we will say that well 1A requires a master mix containing only dTTP, well 2A requires dATP, and well 3A requires dGTP. The liquid handling robot would then transfer by a one step liquid transfer event from e10, e11 e12 from master plate P1 to wells a1, a2, a3 of the reaction plate to add the monomers required for the first DNA synthesis cycle. The next DNA synthesis cycle would then add the next respective nucleotide for each gene. For the sake of providing one more example lets say the amp gene needs a guanine, the phosphatase needs a guanine and the GFP gene needs a thymine. This would require a liquid transfer step from h4, h5, and h6 from master plate P2 to wells a1, a2, and a3 of the reaction plate.

This system is limited to 3-4 additions at a time since adding 2 at a time can be easily improved on by adding 3 or 4 at a time. Adding more than 4 at a time is counterproductive. For example, if you attempt to add 5 wells at a time you would require 20 96 well master plates. Adding 4 wells at a time requires 8 96 well master plates or 2 384 well master plates. If a 384 well plate format is established then only conducting 96 reaction at a time is recommended since conducting all 384 reactions would add to the total cycle time over industry standards and thus be counterproductive. This system makes the use of a liquid handling robot feasible since the total amount of time required to add monomers to a reaction plate is decreased by at least 3x. The monomer addition stem for 96 reactions where monomers are added one well at a time takes 16 mins. Using the Theta+ liquid transfer system, there are 32 liquid transfer events for adding a monomer per cycle cutting the time down to 5.3 mins. This reduces the total cycle time well below the 10 min per cycle industry benchmark for producing 350nt oligos by chemical synthesis.

The first table below shows all possible 3 bp combinations, 64 in total. The second table shows the first 32 combinations distributed in one 96 well plate with one nucleotide per well. And the third table below shows the second 32 combinations set up in the same way as the second table. These tables depict how master plates for adding individual monomers into individual wells of a reaction plate 3 wells at a time should be set up for use in the Theta+ N liquid Handling System for RadegenBio+ DNA Forging.

64 3bp combinations

	1	2	3	4	5	6	7	8	9	10	11	12
a	AAA	AAT	AAC	AAG	CAA	CAT	CAC	CAG				
b	ATA	ATT	ATC	ATG	CTA	CTT	CTC	CTG				
c	ACA	ACT	ACC	ACG	CCA	CCT	CCC	CCG				
d	AGA	AGT	AGC	AGG	CGA	CGT	CGC	CGG				
e	TAA	TAT	TAC	TAG	GAA	GAT	GAC	GAG				
f	TTA	TTT	TTC	TTG	GTA	GTT	GTC	GTG				
g	TCA	TCT	TCC	TCG	GCA	GCT	GCC	GCG				
h	TGA	TGT	TGC	TGG	GGA	GGT	GGC	GGG				

Master Plate P1

p1	1	2	3	4	5	6	7	8	9	10	11	12
a	A	A	A	A	A	T	A	A	C	A	A	G
b	A	T	A	A	T	T	A	T	C	A	T	G
c	A	C	A	A	C	T	A	C	C	A	C	G
d	A	G	A	A	G	T	A	G	C	A	G	G
e	T	A	A	T	A	T	T	A	C	T	A	G
f	T	T	A	T	T	T	T	T	C	T	T	G
g	T	C	A	T	C	T	T	C	C	T	C	G
h	T	G	A	T	G	T	T	G	C	T	G	G

Master Plate P2

p2	1	2	3	4	5	6	7	8	9	10	11	12
a	C	A	A	C	A	T	C	A	C	C	A	G
b	C	T	A	C	T	T	C	T	C	C	T	G
c	C	C	A	C	C	T	C	C	C	C	C	G
d	C	G	A	C	G	T	C	G	C	C	G	G
e	G	A	A	G	A	T	G	A	C	G	A	G
f	G	T	A	G	T	T	G	T	C	G	T	G
g	G	C	A	G	C	T	G	C	C	G	C	G
h	G	G	A	G	G	T	G	G	C	G	G	G